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(54) Title: CLONING AND CHARACTERIZATION OF A GENE, INDUCIBLE BY CYTOKINES

(57) Abstract

The present invention relates to a previously unknown gene, apparently belonging to the pentaxin family, which is inducible by one or more cytokines, and whose expression is not limited to hepatocytes, the mRNA being abundant in cytokine-activated fibroblastic and endothelial cells, and the gene being strongly induced by TNFα and IL-1, but not IL-6 (the primary inducer of SAP and CRP), so that assaying the expression product may provide an independent indication of tissue stress in most tissue types.

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CLONING AND CHARACTERIZATION OF A GENE, INDUCIBLE BY CYTOKINES

The present invention relates to a gene inducible by one or more cytokines.

The pentaxin family of genes is represented right across the animal kingdom, and is remarkably well conserved, even between species as evolutionarily far apart as the horseshoe crab and man [Woo, P. et al., J. Biol. Chem. (1985), 260:13384]. The pentaxins, or pentraxins, are so named because of their pentameric structure [Pepys, M.B., and Baltz, M.L., Adv. Immunol. (1983), 34:141].

The pentaxins fall into that category of proteins designated as "acute phase response proteins". Thus, they are characteristically activated under acute phase conditions, such as tissue stress, inflammation and/or infection.

Typical and well known members of the pentaxin family are C-reactive protein (CRP) and serum amyloid P component (SAP). The existence of these proteins has been well characterised, but their function remains unclear. However, these two particular proteins are known to be induced by various cytokines, particularly interleukin 6 (IL-6), interleukin 1 (IL-1) and tumour necrosis factor alpha (TNFα).

In recent years, it has become clear that many events associated with the "acute phase response" are the consequence of the interaction of cytokines with several target organs. In the case of CRP and SAP, these are particularly IL-6, IL-1 and TNFα. The most

important target organ is the liver [Yap, S.H., et al., Bioch. et Bioph. Acta. (1991), 1091:405]. These cytokines, IL-6 in particular, appear to directly regulate the production of CRP and SAP by acting on their 5' regulatory sequences [Li, S.P., et al., J. Biol. Chem. (1990), 265:4136; Ganter, U., et al., EMBO J. (1989), 8:3773; and Majello, B., et al., EMBO J. (1990), 9:457].

In man, elevated levels of CRP can be detected in the circulation as a result of acute phase conditions, and can be used to monitor the progress of a patient clinically. In the mouse, on the contrary, SAP is capable of providing the diagnostic acute phase reactant, while elevated levels of CRP are not detected [Tillett, W.S., and Francis, T., J. Exp. Med. (1930), 52:561, and Abernethy, T.J., and Avery O.T., J. Exp. Med. (1941), 73:173].

We have now identified a previously unknown gene, which we believe to be of the pentaxin family, which is inducible by one or more cytokines, and whose expression is not limited to hepatocytes.

This new gene, which we have named PTX3, has been cloned from a cDNA library constructed by poly-A^+ selected mRNA from human umbilical vein endothelial cells (HUVEC) exposed for 1 hour to interleukin-1. The library was differentially screened with cDNA probes from untreated versus IL-1-treated HUVEC, as detailed below, in an effort to clone and characterize genes whose transcription may be specifically induced by the exposure to IL-1.

Thus, in a first aspect, the present invention

provides a nucleotide sequence corresponding to that of Sequence ID n. 1, from nucleotide 57 to nucleotide 1148 inclusive, and mutants and variants thereof.

The present invention also provides characteristic portions of the above sequence, and sequences comprising the above sequence, especially those sequences including those nucleotides corresponding to positions 6 to 56 in the Sequence ID n. 1.

By "characteristic" portions is meant those sequences which are unique to the sequence of the invention.

Nucleotides 6 to 56 almost certainly encode a signal peptide and, so, are not essential to the expression product.

Nucleotides 57 to 1148 inclusive, code for an expression product. The putative amino acid sequence of the gene is also shown in Figure 3, which extends from methionine 1 (nucleotides 6 to 8) to serine 381 (nucleotides 1146-1148). The sequence appears to constitute one open reading frame, which codes for a protein of approximately 42 kd as proved by in vitro transcription-translation of the full lenght cDNA clone, when subcloned in an appropriate expression vector (Figure 4) as detailed below.

The expression product encoded by the sequence of the invention may also be encoded by other sequences, due to the degeneracy of the genetic code. Such degenerate sequences are termed "mutants" herein. It will also be appreciated that the same gene may vary between species or even intraspecies. Thus the term "variants" includes species variants as well as allelic

variants.

By computer analysis, we have established that the expression product of the sequence of the invention is probably a member of the pentaxins. This is confirmed by the high degree of homology between parts of the putative protein sequence and the pentaxins, as well as the possession of the characteristic pentaxin signature [Bairoch, A., Nucl. Acid Res. (1991), 19: 2241].

This is shown in Figure 3 by the alignement of the amino acid sequences of the two known members of the pentaxin family with each other and with PTX3.

The characteristic peptide is also shown in Fig. 3 by underline and corresponds to the aminoacid sequence HLCGTWNS extending from aminoacid 269 to aminoacid 276 included.

Experimentation has also shown that, upon exposure to several cytokines, the gene is expressed in cell lines other than hepatocytes, endothelial and fibroblasts and so is of more general use as an indicator of stress conditions than CRP in man (Figure 4).

A polyclonal antibody was produced in the rabbit against a synthetic decapeptide corresponding to amino acid sequence 272-281 inclusive (Gly-Thr-Trp-Asn-Ser-Glu-Glu-Gly-Leu-Thr) of the Sequence ID n. 1. This antibody specifically recognises the in vitro transcribed and translated PTX3 (data not shown) and, more significantly, is able to immunoprecipitate a protein of approximately 42 Kd from the supernatant of IL-1 treated HUVEC (Figure 5). These data prove that PTX3 is indeed produced in cytokine-stimulated cells

and released in the supernatant as hypothized by the presence of the signal peptide.

Accordingly, the present invention provides the expression product of the above sequence, including variants thereof, and characteristic partial peptides of the expression product, wherein "characteristic" has the meaning defined above.

There are further provided conjugate sequences comprising the peptides of the invention. Such sequences may comprise a leader sequence for an heterologous host for example.

Both prokaryotic and eukaryotic host can be used for the expression of PTX3 sequences. For istance, the fragment of Sequence ID n. 1 from nucleotide 253 to nucleotide 1775, previously identified as PTX3/C (Figure 1) has been subcloned into the prokaryotic expression vector pET3C to obtain large amounts of recombinant protein.

The full length cDNA of PTX3 as disclosed by Breviario F. et al., J. Biol. Chem. 1992, 267, 31, 22190-22197) has been subcloned into the eukaryotic expression vector pSG5 to obtain large amounts of recombinant protein from the surnatant of transfected COS cells.

The invention also provides several methods for assaying PTX3. This may be either direct or indirect. An indirect assay may comprise assaying mRNA, in which case a probe corresponding to a sequence of the invention may be used. Such probes will be sense probes, but the invention also contemplates antisense probes. In addition, the probes may be DNA or RNA

probes as preferred, and may be synthetic or cloned, as desired.

If the detection method is direct, then it is preferred to employ antibodies to the expression product.

The invention provides information on the production of antibodies, able to specifically recognize the protein product of PTX3.

The advantage of the present invention is that the tissue type is not important, as PTX3 mRNA is abundant in cytokine-activated fibroblastic, hepatic and endothelial cells. Thus, stress in most tissue types may be assessed by assaying PTX3.

In addition, PTX3 is strongly induced by TNF and IL-1, but not IL-6 (the primary inducer of SAP and CRP), so that assaying this protein may also provide an independent indication of tissue stress etc.

Finally, PTX3 is clearly induced and released in supernatant by TNF treatment of 8387 cell line.

The invention will now be further illustrated by the following Example.

EXAMPLE

Cell culture and stimulation:

HUVEC were cultured and characterised as described previously in detail [Dejana, U., et al., J. Cell. Biol. (1988), 107:1215] and used at the 3rd-7th passage. The hepatoma cell line HEP 3B was cultured in Dulbecco's modified Minimal Essential Medium supplemented with 15% fetal calf serum (FCS, Gibco, Paisley, Scotland). The fibrosarcoma cell line 8387 was cultured in Minimal Essential Medium and 10% FCS.

The COS cell line was maintained in D-MEM +10% FCS + Na piruvate.

Cells for RNA extraction were grown to confluence in 75cm^2 flasks. Just before stimulation, the culture medium was removed and the cells were washed twice with calcium- and magnesium-free Hank's Balanced Salt solution (Gibco). 7ml of endotoxin-free RPMI1640 (Gibco) supplemented with 5% FCS and 20mg/ml Polimixin-B sulphate (Sigma, St. Louis, MO) was then added with or without the indicated cytokines. Human recombinant IL-1ß (Boehringer, Mannheim) was used at 20 ng/ml final concentration and human recombinant TNFα (BASF/Knoll, Ludwigshafen, Germany) at 500 U/ml. Human recombinant Il-6 (Boehringer, Mannheim) was used at 50U Cess/ml. At these concentrations IL-1ß and TNFα were able to induce biological effects on HUVEC such as neutrophil adhesion, procoagulant activity and prostacyclin production [Dejana, E., et al., Blood (1987), 69:695].

RNA extraction and Northern blot analysis:

Total cytoplasmic RNA was extracted and purified as previously described [Golay, J., et al., Blood (1991), 77:149]. Ten µg of total cellular RNA were run in standard formaldehyde/agarose gels, blotted on nitrocellulose membranes (Schleicher and Schuel, Dassel, Germany) and fixed under vacuum at 80°C for 2 hours. Plasmid probes were labelled with ^{32}P-dCTP (Amersham, Bucks, England) by standard nick-translation procedures to specific activities of 1-2 x 10^8 cpm/µg DNA. Hybridisations were performed as described previously [Sambrook, J., et al., Cold Spring Harbor Laboratory (1989)].

Construction of cDNA library and differential screening:

Total RNA was isolated from HUVEC cultured for 1 hour in the presence or absence of both IL-1ß and cycloheximide at 10 µg/ml. Poly(A^+) RNA was further purified by affinity chromatography on oligo(dT)-cellulose [Tabor S. and Richardson C.C., Proc. Natl. Acad. Sci. USA (1987), 84:4767].

A cDNA library was constructed in the lambda-ZAP vector (Stratagene, La Jolla, CA) as described [Tabor S. and Richardson C.C., Proc. Natl. Acad. Sci. USA (1987), 84:4767]. 4000 recombinant clones were screened by differential hybridisation with single stranded cDNA probes complementary to mRNA from untreated or IL-1 treated HUVEC. To generate the cDNA probes, 2 µg of poly(A^+) RNA were denatured at 65°C for 10 minutes and then put on ice. The labelling reaction was performed in a 10 µl final volume in 1x reverse transcriptase buffer (8 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 50 mM Tris pH8.1) containing 4 mM sodium pyrophosphate, 0.5 µl RNAse inhibitor (40U/ml, Promega Corp., Madison, WI), 0.01 µg oligo-dT (Pharmacia, Uppsala, Sweden), 100 µM each of d(A,G,T)TP, 250 µCi of ^{32}P-dCTP (Amersham, <3000Ci/mmole) and 0.5 ml of AMV reverse transcriptase (7U/ml, Promega). After 10 minutes at 42°C, a further 1 µl of dNTP (10 mM each) was added and the reaction allowed to proceed at 42°C for further 20 minutes.

The RNA strand was hydrolysed by the addition of 10 µl of 0.3 M NaOH, 30 mM EDTA, boiling for 5 minutes and then neutralising with 2.5 µl of 1M Tris-HCl, pH8.

The cDNA probe was separated from free nucleotides by chromatography through a 5 ml G-50 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) column, yielding about 1.5×10^8 cpm cDNA probe/µg mRNA.

For the differential screening, the library was plated at approximately 1 plaque/cm^2 and transferred onto nitrocellulose membranes in duplicates by standard procedures [Sambrook J. et al., Cold Spring Harbor Laboratories, N.Y.]. The membranes were hybridised at 42°C for 48 hours in 0.02M Tris-HCl pH7.6, 50% deionised formamide, 5 x SSC, 1x Denhardt's solution, 10% Dextran sulphate, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA and with 1×10^7cpm/100cm^2 of cDNA probe. Membranes were washed at 50-60°C in 0.2 x SSC, 0.1% SDS. Plaques showing induction with treated relative to untreated HUVEC mRNA were picked and rescreened three times differentially in order to obtain single clones.

Sequence analysis:

Sequencing of the Bluescript plasmids containing the phage inserts was performed with the dideoxynucleotide chain termination method [Tabor S. and Richardson C.C., Proc. Natl. Acad. Sci. USA (1987), 84:4767] and Sequenase enzyme (United States Biochemical Corporation, Cleveland, OH).

Computer methods:

The sequences were screened against the EMBL Nucleotide Sequence database [Stoehr, P.J. and Cameron G.N., Nucl. Acids Res. (1991), 19:2227] using the FASTA programme [Pearson W.R. and Lipman D.J., Proc. Natl. Acad. Sci. USA (1988), 1985:2444] as implemented on the

EMBnet computer resource [Stoehr, P.J., Omond R. eds, 1991], screening of the SWISS-PROT protein sequence data bank [Bairoch A. and Boeckmann B., Nucl. Acids Res. (1991), 19:2247] and detailed sequence analysis was carried out with the PC/Gene sequence analysis package (IntelliGenetics).

In vitro transcription-translation:

The BAM HI-KPNI frangment from PTX3/D cDNA clone, containing the entire open reading frame, was subcloned in PGEM3 vector (Figure 7). Upon linearization with KPNI, the DNA has been transcribed in vitro by SP6 RNA polimerase and translated into protein upon addition of wheat germ agglutinin (Promega) in the presence of 35S-methionine, according to the manifacturers instructions (Promega). The protein product was then analysed in standard SDS-PAGE analysis. Occasionally, the same in vitro translation reaction was conducted in the absence of 35S-methionine and the protein product upon SDS-PAGE was electroblotted onto nitrocellulose filters for subsequent western analysis.

Western blotting and immunoprecipitation

SDS-PAGE and electroblotting in Tris-glycine buffer were performed according to standardised methods (Molecular Cloning: A Laboratory Manual; Second Edition. Sambrook, Fritsch, Maniatis eds, Cold Spring Harbor Laboratory Press).

The membranes were pre-incubated in PBS + 3% BSA (SIGMA) for 1 h, R.T., and then hybridized with the antibody diluitions for different time intervals. Finally the membranes were washed and incubated with anti-rabbit Ig horseradish peroxidase linked whole

antibody (Amersham) for 4 h at room temperature.

Blots were then washed again and detection of labelled protein was performed by enhanced chemiluminescence (ICL, Amersham) according to the manufacturers instructions.

Following incubation in the presence of 35S-metionine, the cell supernatants are concentrated and then incubated in the presence of the antiserum and cold G protein for 1 h at room temperature. Finally the complexes are precipitated by centrifuge spinning and washed several times and then loaded for SDS-PAGE analysis.

Peptides

The complete amino acid sequence of PTX3 was analysed for hydropathy plot using Mac Molly Computer Programme. Based on the most hydrophilic regions, several peptide were syntetized on solid phase with the 430 A peptide synthesizer by Applied Biosystem. Some peptides were conjuagated with bovine albumine (glutaraldheide), some with haemocyanin, following introduction of the sequence Gly-Gly-Cys in the COOH end of the peptide.

Polyclonal antibodies production

Rabbits were first immunized with 100 µg of the synthetic decapeptide resuspended in Freund's complete adjuvant and boosted at intervals of 2-3 weeks with the same amount of peptide. All injections were performed subcutaneously. Rabbits were bled repeatedly and sera analysed by standard Elisa assays against the variuos peptide preparations in microwell plates.

The polyacrilamide gel band containing the protein

expressed by the PTX3C sequence as indicated by the staining, was excised from the gel, mechanically disrupted using a needle and a syringe and injected subcutaneously into a 28 days old rabbit (Charles River, Calco, Italy). This procedure was repeated three times at intervals of 4 weeks and then the animal was bleeded and the serum used as a source of polyclonal anti PTX3 antibody in Western analysis.

Subcloning of PTX3 into pET 3c vector

The PTX3C cDNA into Bluescript vector (Breviario F. et al., J. Biol. Chem., 1992, 267, 22190-22197) was cut with Kpn I, blunted with Klenow DNA Polymerase and riclosed in the presence of a molar excess of the oligonucleotide CGCGGATCCGCG containing an internal Bam Hl site. Unless differently specified, all the standard protocols used including the chemical composition of the buffers are according to: "Molecular cloning: a laboratory manual". II edition, Eds.: Sambrook, Fritch, Maniatis. Cold Spring Harbor, 1989. Following transformation of competent HB101 cells with the ligation mix, we isolated 1 recombinant construct, named PTX3(-250)" originated from PTX3C in which the Kpn I site had been substituted with a Bam Hl site.

The recombinant construct was digested with Bam Hl and the fragment of 1608 bp isolated on 1% Agarose gel in TAE buffer and purified by Gene Clean (Bio 101 La Jolla, CA, USA) in accordance with the manufacturer's instructions.

The fragment was ligated into the Bam Hl site of pET 3c plasmid in the presence of T4 DNA ligase and the ligation mix was used to transform competent HB101

cells.

One recombinant clone "pET 3c + PTX3 (-250)" was selected by sequence analysis to verify the reading frame, and was used for the expression of PTX3 protein in prokaryotic cells (Figure 8).

Expression of "PTX3 (-250)" in prokaryotic cells

BL21 bacteria were grown in NZCYM and made competent in accordance with standard procedures. Competent cells were transformed with 20-50 ng of "pET 3c + PTX3 (-250)" or "pET 3c" alone, plated on 1.5% Agar in Luria Broth medium and grown overnight at 37°C. Colonies were expanded in NZCYM for 4-5 h at 37°C and then induced with 0.6 mM IPTG (Sigma, St. Louis, MO, USA) for 4 h. At the end of incubation, induced bacteria were pelletted for 15' at 3000 x g and the supernatant was discarded. Pellets were dissolved in sonication buffer (300 mM KCl, 20 mM Hepes, 0.1 mM EDTA, 1 mM DTT, 0.05% NP40) and sonicated for 10' in ice, then ultracentrifuged at 35000 RPM for 30'. This sonication step was repeated one more time and finally the pellets were sonicated in 8M Urea and stored refrigerated until analysed on polyacrilamide gels (Figure 9).

Subcloning of PTX3/E cDNA into pSG5 vector

The PTX3/E cDNA into Bluescript vector (Breviario F. et al., J. Biol. Chem., 2992, 267, 22190-22197) was cut with Hind III. Due to the presence of one Hind III site at position 1337 of the PTX3/E insert and to the presence of another Hind III site into the polilinker of the Bluescript vector (Figure 10A) upon digestion and completion, we separated two fragments on 0.8%

agarose gel in TAE buffer. The major fragment (containing the vector) was isolated with Gene Clean (Bio 101) according to the manufacturer's instructions and riclosed on itself in the presence of T4 DNA ligase (Promega, Madison, WI, USA). Upon transformation of competent E. Coli, strain HB101, we identified one recombinant plasmid, "PTX3 1.3", which was purified and amplified with standard procedures (Figure 10B). Subsequently "PTX3 1.3" was digested with Kpn I, which cuts only once in the polilinker and its termini were blunted with Klenow DNA polymerase according to standard protocols. Finally, the plasmid was riclosed on itself in the presence of a molecular excess of the oligonucleotide CGCGGATCCGCG containing an internal Bam Hl site. Upon transformation with the ligation mixture, we then isolated one recombinant construct, "PTX3 1.3 Bam/Bam", in which the Kpn I site had been substituted with a Bam Hl site (Figure 11). Following purification and amplification with standard protocols, we finally digested "PTX3 1.3 Bam/Bam" with the Bam Hl enzyme in order to isolate the fragment of 1472 bp containing the PTX3 cDNA sequence. This fragment, upon Gene Clean purification, was ligated into the pSG5 vector which had been previously digested with Bam Hl and dephosphorilated with standard procedures to give the recombinant plasmids "PTX3 1.3 sense" and "PTX3 1.3 antisense" relative to the two possible orientations of the insert, as schematized in Figure 12, 13.

<u>Transfection of PTX3 1.3 sense and PTX3 1.3 antisense into COS cells</u>

COS cells were maintained in colture as previously

described. Petri dishes were seeded with $2x10^5$ COS cells in DMEM with 10% FCS and incubated at 37°C overnight. The next day, 20 µg of "PTX3 1.3 sense" or "antisense" were precipitated with standard calcium phosphate method and the aggregates were left for 18 h. The monolayers were then washed extensively with saline and incubated in DMEM w/o FCS for additional 6 to 18 h. At the end of this period, the supernatants were collected, spun at 2000 RPM for 10' and concentrated approximately 10 times with Centricon 10 (Amicon, W.R. Grace & Co., MA, USA).

Supernatant from mock-transfected cells or COS transfected with sense or antisense constructs, were analysed on a 10% polyacrylamide gel under reducing conditions, elettrotransferred to nitrocellulose and hybridized with rabbit polyclonal antibody anti PTX3 obtained as previously described by immunization of the animals with the protein expressed by the PTX3C sequence (Figure 14).

Expression in 8387 cells

8387 Human sarcoma cells were grown in MEM with 10% FCS. When cells were subconfluent, medium was removed and the monolayer extensively washed with saline. Cells were then incubated in MEM w/o FCS in the presence of 500 U/ml human recombinant TNF (BASF/knoll, Ludwighafen, Germany) for 6-18 h at 37°C. At the end of incubation, supernatants were removed from control or TNF-stimulated cells, spun at 2000 rpm for 10' at 4°C, concentrated and analysed by SDS-PAGE followed by Western Blot as previously described (Figure 15).

Isolation and characterization of Sequence ID n. 1

A cDNA library was constructed from poly(A^+) mRNA purified from HUVEC stimulated with IL-1ß for 1 hour in the presence of cycloheximide.

Over 4000 separate clones were differentially and repeatedly hybridised with ^{32}P-labelled cDNAs from untreated versus IL-1 stimulated HUVEC. 38 separate clones which gave a stronger signal when hybridised with the "induced" probe were selected and partially sequenced (300-400 nucleotides). 36 clones corresponded to genes already cloned and known to be induced IL-1 in endothelial cells, that is Il-8 (16 clones) [Sica A., et al., Immunology (1990), 69:548], endothelial leukocyte adhesion molecule-1 (ELAM-1) (7 clones) [Bevilacqua M.P., et al., Science (1989), 243:1160], Gro-α (6 clones) [Wen D., et al., EMBO J. (1989), 8:1761], Gro-ß (5 clones) [Haskill S., et al., Proc. Natl. Acad. Sci. (1990), 87:7732] and plasminogen activator inhibitor (PA-i) (2 clones) [Gramse M., et al., Bioch. Bioph. Res. Comm. (1986), 139:720].

The remaining two clones contained an identical sequence of approximately 900 base pairs which did not correspond to any known sequence present in the EMBL Nucleotide Sequence Database and are schematically represented in Figure 1 as PTX3/A. PTX3/A contains an open reading frame starting at its 5' end (EcoRI site) and a TAA stop codon, followed by a long 3' untranslated region including a polyadenylation signal.

In order to try and clone the full length cDNA, another cDNA library was screened with PTX3/A as probe and several additional clones were found which are schematically represented in Figure 1 as PTX3/B, C and

D, respectively. The longest (PTX3/D) contains an insert of 1775 bp, which is quite close to the size of the mature message as detected in Northern blots and estimated to be of approximately 1.8kb (see below), since it almost exactly overlaps the 18S ribosomal RNA. The different clones were completely sequenced in both orientations as schematised by arrows in Figure 1.

More recently the full lenght cDNA clone was isolated (PTX3/E), containing 62 additional nucleotides as 5' intranslated flanking sequence (Breviario F. et al., J. Biol. Chem. 267, 1992, 22190-22197). These additional nucleotides are identified with negative number from -62 to +1, which coincides wiht the first nucleotide of PTX3/D (Figure 1).

In Figure 1, a map is shown of PTX3 cDNA clone (PTX3/D) showing some unique restriction sites. The arrows represent the extent and direction of sequencing for the PTX3/D clone. In the lower part the other types of PTX3 clones obtained (PTX3/A-C) in relation to PTX3/D are shown.

Figure 1 shows the complete nucleotide and amino acid sequence of PTX3 cDNA. The nucleotides are numbered from 1 to 1775 on the left-hand side. The longest open reading frame and its translated protein sequence are shown. The amino acids are numbered from 1 to 381 on the right-hand side. The potential signal peptide of 17 amino acids starting with the first methionine is underlined. The arrow at the centre of the sequence indicates the cysteine residue where the homology with the pentaxins begins. The dotted line underscores the three amino acids which constitute a

potential N-glycosylation site. The double line underlines the eight amino acids which constitute the "pentaxin family signature". The polyadenylation signal spans nucleotides 1740 to 1745.

The entire sequence spanning from nucleotide 1 to nucleotide 1775 has been deposited on the 15 January 1992 at the National Collection of Type Cultures London NWS SHT according the Budapest Treaty with number NCTC 12498 in the Bluescript Plasmid carried by the E. coli strain HB101. Figure 6 is a schematic representation of this ricombinant plasmid.

The sequence shown in Sequence ID n. 1 has an open reading frame from the 5' end to the codon at position 1149.

The predicted protein sequence of 381 amino acids is shown, and this has a theoretical unglycosylated molecular weight of 42 kD.

In order to confirm that the PTX3/D cDNA contained the predicted open reading frame, the BAMHI-KPNI fragment was subcloned in PGEM3 vector (Figure 2). The in vitro transcription-translation protein product shows on SDS-PAGE analysis the expected 42 kd molecular weight (Figure 7).

Screening the complete nucleotide sequence against the EMBL Nucleotide Sequence Database revealed that the region spanning nucleotides 1 to 831 (the EcoRI site) is nearly completely colinear (98% homology) with a sequence corresponding to a partial cDNA sequence of a TNF inducible gene, called TSG-14a (accession number M31166), isolated from human fibroblasts [Lee T.H., et al., Mol. Cell. Biol. (1990), 10:1982]. The M31166

sequence is 70 nucleotides longer at its 5' end compared to PTX3/D, and contains 13 differences in the 828 overlapping nucleotides.

The protein sequence of 381 residues predicted for the longest PTX3 open reading frame was analysed against the EMBL SWISS-PROT protein sequence data bank using the PC/Gene package. A significant alignment was found between the 3' portion of the PTX3 sequence, from the cysteine at position 179 (marked with an arrow in Figure 1) to the C terminus, and the nine cloned members of the pentaxin gene family.

In Figure 3 there is shown the alignment and degree of conservation between PTX3 and the human CRP and SAP proteins. The full protein sequence of human CRP and SAP is shown, whereas only the region of PTX3 showing similarity to the pentaxins is indicated, from the cysteine at position 179 to the C-terminus. The dots represent conservation, the asterisks identity. The "pentaxin family signature" is doubly underlined.

Figure 3 shows the alignment of the predicted protein sequence of PTX3 with the two human members of the pentaxin family, namely C-reactive protein (CRP) and serum amyloid P component (SAP): 35 out of the 208 amino acids of the consensus region are identical (17%) and 118 are conserved (57%) in all three proteins.

The alignment for PTX3 with the nine cloned pentaxins originating from the mouse, rabbit, hamster and horseshoe crab, in addition to man, shows an overall identity of 22 amino acids over 259 consensus length (9%) and a similarity of 55 amino acids (21%) (data not shown). In particular, there are two

cysteines at amino acid positions 210 and 271 of PTX3 (Figure 3) which are conserved in all members of the family and are thought to play a crucial role in determining the secondary structure of the molecule [Pepys, M.B., and Baltz, M.L., Adv. Immunol. (1983), 34:141]. PTX3, on the other hand, completely diverges from all other pentaxins at its 5' terminus and appears to be longer, in contrast to the other members of the family which are generally conserved throughout their entire sequence and have similar lengths.

The predicted PTX3 amino acid sequence was also screened against the PROSITE protein pattern database [Bairoch, A., Nuc. Ac. Res. (1991), 19:2241] which revealed the presence in PTX3 of the eight amino acids (H-x-C-x-S/T-W-x-S) constituting the "pentaxin family signature". These amino acids are doubly underlined in Figures 1 and 3. The 5' portion of the predicted sequence of PTX3, down to the cysteine residue at position 179 (Sequence ID n. 1), is not apparently significantly related to any known protein sequence.

The similarity of PTX3 to CRP and SAP commences with the second exon of both the CRP and SAP genes [Woo P., et al., J. Biol. Chem. (1985), 260:13384], their first exons, which code for the signal peptides, diverging from the PTX3 sequence.

The total length of the longest cDNA clone (PTX3/D, 1775bp) is very close to the size of the mature message detected by Northern analysis, estimated at 1.8 Kb. The PTX3 open-reading frame contains 6 methionine residues before the start of the pentaxin-like domain (Sequence ID n. 1).

We believe that the methionine residue at amino acid position 1 (Sequence ID n. 1) is that which is effectively used, particulalrly as it fits the Kozak consensus sequence [Kozak M, J. Cell. Biol. (1989), 108:229]. This methionine is immediately followed by a typical signal peptide (underlined in Sequence ID n. 1), as predicted according to the method of von Heijne [von Heijne G., Nuc. Acid. Res. (1986), 14:4683]. The predicted cleavage site for the signal peptide is between the alanine and glutamic acid at positions 17 and 18. This putative signal peptide shows a 9 out of 12 amino acids identity with that of murine and human tyrosinases (SWISS-PROT accession numbers P11344 and P14679), evidence that this sequence actually encodes a signal peptide. In addition, in vitro transcription and translation of the full PTX3/D clone led to the synthesis of a unique 42kD protein, as would be predicted for a protein starting with the first methionine (Figure 7).

PTX3 gene expression in different cell types was then studied by Northern blot analysis. In HUVEC, PTX3 mRNA is strongly induced by IL-1ß and TNFα but not by IL-6 (Figure 4, upper panel). Induction is rapid and transient. The appearance of the message is evident also when IL-1ß is added in the presence of cycloheximide (data not shown), as expected, since the library was constructed following exposure of HUVEC to both IL-1ß and cycloheximide. This suggests that PTX3 is an "early" response gene to IL-1ß. The same pattern of induction to IL-1ß and TNFα but not to Il-6 is also observed in the human hepatoma cell line HEP3B (Figure

4, lower panel), which is an IL-1 and IL-6 responsive cell line. Again, the PTX3 message appears with rapid and transient kinetics.

Both IL-1ß and TNFα are able to induce the expression of PTX3 mRNA in the human fibrosarcoma cell line 8387 (Figure 4, lower panel). In this case, however, the TNFα signal seems to be more effective than IL-1ß in inducing PTX3. This would be in agreement with the fact that the related TSG-14a gene was isolated from another fibroblastic cell line as a TNFα inducible gene [Lee T.H., et al., Mol. Cell. Biol. (1990), 10:1982].

In order to obtain a polyclonal antibody able to detect the PTX3 product, we analysed the putative amino acid sequence with the hydrophobicity plot to look for hypothetical antigenic regions. Several synthetic peptides were made and one of those, namely the decapeptide from Gly at position 272 to Thr at position 281 inclusive, gave positive results.

The antiserum was positive in several Elisa assay, and reacted well with the in vitro transcribed/translated protein product of PTX3. (data not shown).

Finally, HUVEC were metabolically labelled in the absence or in presence of interleukin-1 for 6 h with 35S-methionine and the supernatants from such coltures were immunoprecipitated with the antiserum as detailled above. As shown in Figure 5, a unique band of approximatery 42 kd is visible in the supernatant from untreated cells, which is clearly augmented by the presence of interleukin-1 (Figure 5, lanes 1, 2).

These data further support that PTX3 protein is indeed produced in normal endothelial cells and released in the supernatants, as hypothized by the presence of a complete signal peptide. Furthermore, upon exposure to IL-1, a clear increase in the amount of released PTX3 protein, could be observed.

Another polyclonal antiserum was raised in rabbits, by immunization with a fragment of prokaryotic recombinant PTX3 (PTX3/C) upon subcloning into pET3c vector as schematized in Figure 8 and induction with IPTG and isolation by excision from SDS-PAGE as shown in Figure 9.

This antibody recognizes the full lenght PTX3/E, when subcloned into pSG5 eukaryotic expression vector (the construction of the recombinant is schematized in Figures 10A, 10B, 11, 12 and 13 and described in the examples), in the surnatant of transfected COS cells (Figure 14). Furthermore it recognizes the native PTX3 protein in the surnatant of TNF stimulated human sarcoma 8387 cell line (Figure 15).

All in all, the data stress that PTX3 protein is indeed produced and released in the surnatant of cytokine stimulated cells, where it has been detected by Western analysis.

Detection of PTX3 in human serum

In order to investigate whether PTX3 is secreted in the blood stream during pathological processes, we tested serum samples obtained from patients and healthy donors randomly selected.

The PTX3 presence in serum was determined by an enzyme linked immunoassay (ELISA) carried out with

rabbit polyclonal antibody raised against PTX3 (-250). For this purpose rabbit immunoglobulins were purified from serum by affinity chromatography on Sepharose Protein A (Pharmacia) according to the manufacturer's instructions. Subsequently purified immunoglobulins were labelled with biotin as follows: a 10 times molar excess of NHS-Biotin (Pierce) (10 mg/ml in DMF) was added to immunoglobulin solution (2 mg/ml in 20 mM borate buffer pH 8.5 containing 150 mM NaCl). The reaction mixture was incubated 20 min at room temperature before adding 1/10 in volume of 1M NH_4Cl pH 7.5 Biotinylated immunoglobulins were then extensively dialyzed against PBS.

For ELISA tests, 96-well plates were coated over night at 4°C with rabbit immunoglobulins anti-PTX3 (50 µg/ml) in 0.1 M carbonate buffer pH 9.6, then blocked with 3% BSA (Bovine Serum Albumin) in PBS for 1 h at 37°C. Plates were incubated over night at 4°C with 100 µl of serum sample diluted in PBS. After three washes, biotinylated immunoglobulins (10 µg/ml) were added and the plates incubated 1 h at 37°C. After 3 washes, alkaline phosphatase-labelled avidine (Zymed) was added (15 min at 37°C). The reaction was developed, with p-nitrophenyl-phosphate 1 mg/ml in 0.1 M diethanolammine buffer pH 9.8 (1 h at 37°C). The plates were read by microplate reader (Perkin Elmer).

Out of 25 patients with different pathologies the presence of PTX3 was detected in 30-40% of the cases; all healthy donors were negative.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Italfarmaco
(B) STREET: F.Testi
(C) CITY: Milan
(E) COUNTRY: Italy
(F) POSTAL CODE (ZIP): 20100

(ii) TITLE OF INVENTION: CLONING AND CHARACTERIZATION OF A GENE INDUCIBLE BY CYTOKINES

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1775 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CAGCAATGCA TCTCCTTGCG ATTCTGTTTT GTGCTCTCTG GTCTGCAGTG TTGGCCGAGA 60

ACTCGGATGA TTATGATCTC ATGTATGTGA ATTTGGACAA CGAAATAGAC AATGGACTCC 120

ATCCCACTGA GGACCCCACG CCGTGCGCCT GCGGTCAGGA GCACTCGGAA TGGGACAAGC 180

TCTTCATCAT GCTGGAGAAC TCGCAGATGA GAGAGCGCAT GCTGCTGCAA GCCACGGACG 240

ACGTCCTGCG GGGCGAGCTG CAGAGGCTGC GGGAGGAGCT GGGCCGGCTC GCGGAAAGCC 300

TGGCGAGGCC GTGCGCGCCG GGGGCTCCCG CAGAGGCCAG GCTGACCAGT GCTCTGGACG 360

AGCTGCTGCA GGCGACCCGC GACGCGGGCC GCAGGCTGGC GCGTATGGAG GGCGCGGAGG 420

CGCAGCGCCC AGAGGAGGCG GGGCGCGCCC TGGCCGCGGT GCTAGAGGAG CTGCGGCAGA 480

CGCGAGCCGA CCTGCACGCG GTGCAGGGCT GGGCTGCCCG GAGCTGGCTG CCGGCAGGTT 540

GTGAAACAGC TATTTTATTC CCAATGCGTT CCAAGAAGAT TTTTGGAAGC GTGCATCCAG 600

TGAGACCAAT GAGGCTTGAG TCTTTTAGTG CCTGCATTTG GGTCAAAGCC ACAGATGTAT 660

TAAACAAAAC CATCCTGTTT TCCTATGGCA CAAAGAGGAA TCCATATGAA ATCCAGCTGT 720

ATCTCAGCTA CCAATCCATA GTGTTTGTGG TGGGTGGAGA GGAGAACAAA CTGGTTGCTG 780

AAGCCATGGT TTCCCTGGGA AGGTGGACCC ACCTGTGCGG CACCTGGAAT TCAGAGGAAG 840

GGCTCACATC CTTGTGGGTA AATGGTGAAC TGGCGGCTAC CACTGTTGAG ATGGCCACAG 900

GTCACATTGT TCCTGAGGGA GGAATCCTGC AGATTGGCCA AGAAAAGAAT GGCTGCTGTG 960

TGGGTGGTGG CTTTGATGAA ACATTAGCCT TCTCTGGGAG ACTCACAGGC TTCAATATCT 1020

GGGATAGTGT TCTTAGCAAT GAAGAGATAA GAGAGACCGG AGGAGCAGAG TCTTGTCACA 1080

TCCGGGGGAA TATTGTTGGG TGGGGAGTCA CAGAGATCCA GCCACATGGA GGAGCTCAGT 1140

ATGTTTCATA AATGTTGTGA AACTCCACTT GAAGCCAAAG AAAGAAACTC ACACTTAAAA 1200

CACATGCCAG TTGGGAAGGT CTGAAAACTC AGTGCATAAT AGGAACACTT GAGACTAATG 1260

AAAGAGAGAG TTGAGACCAA TCTTTATTTG TACTGGCCAA ATACTGAATA AACAGTTGAA 1320

GGAAAGACAT TGGAAAAAGC TTTTGAGGAT AATGTTACTA GACTTTATGC CATGGTGCTT 1380

TCAGTTTAAT GCTGTGTCTC TGTCAGATAA ACTCTCAAAT AATTAAAAAG GACTGTATTG 1440

TTGAACAGAG GGACAATTGT TTTACTTTTC TTTGGTTAAT TTTGTTTTGG CCAGAGATGA 1500

ATTTTACATT GGAAGAATAA CAAAATAAGA TTTGTTGTCC ATTGTTCATT GTTATTGGTA 1560

TGTACCTTAT TACAAAAAAA ATGATGAAAA CATATTTATA CTACAAGGTG ACTTAACAAC 1620

TATAAATGTA GTTTATGTGT TATAATCGAA TGTCACGTTT TTGAGAAGAT AGTCATATAA 1680

GTTATATTGC AAAAGGGATT TGTATTAATT TAAGACTATT TTTGTAAAGC TCTACTGTAA 1740

ATAAAATATT TTATAAAACT AAAAAAAAAA AAAAA 1775

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO Italfarmaco S.p.A.
Sede e Stabilimento
20126 MILANO
Viale Fulvio Testi 330
ITALY

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Dr D.G. Broccheti Address: Italfarmaco S.p.A. Sede e Stabilimento 20126 MILANO Viale Fulvio Testi 330 ITALY	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 12498 Date of the deposit or of the transfer: 15th January 1992

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 22nd January 1992[2]. On that date, the said microorganism was

[X][3] viable

[ ][3] no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

Form BP/9 (first page)

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

Luria-Bertani Agar + 100mg/ml ampicillin
and
Nutrient Agar
Viability in excess of $20x10^6$ cfu/ml (24hrs, 37°C, Aerobic)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: National Collection of Type Cultures
Central Public Health Laboratory

Address: 61 Colindale Avenue
London, NW9 5HT

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 9th April 1992 L.R. Hill

L.R. Hill, Curator NCTC

[4] Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

1. A nucleotide sequence comprising from nucleotide 56 to nucleotide 1148 inclusive of the Sequence ID n. 1, and mutants and variants thereof.

2. A nucleotide sequence comprising from nucleotide 253 to nucleotide 1775 inclusive, and mutants and variants thereof.

3. A nucleotide sequence according to claim 1, said sequence including those nucleotides corresponding to positions 6 to 55 in the accompanying Sequence ID n. 1.

4. A nucleotide sequence according to claim 3, said sequence encoding a functional signal sequence.

5. A vector comprising a sequence according to any preceding claim.

6. A host cell comprising a vector according to claim 5.

7. An expression system for a vector according to claim 5, comprising cells as defined in claim 6.

8. A method for obtaining an expression product of a sequence according to any of claims 1 to 4, comprising cultivating cells as defined in claim 6, and collecting the expression product.

9. An expression product corresponding to a sequence according to any of claims 1 to 4.

10. A conjugate peptide sequence comprising a peptide according to claim 9.

11. A sequence according to claim 10, comprising a leader sequence for an heterologous host.

12. A method for assaying an expression product or sequence as defined in any preceding claim, comprising

use of an antisense nucleotide sequence or an antibody, as appropriate.

13. A nucleotide sequence having the pentaxin signature and which corresponds to naturally occurring DNA, the naturally occurring DNA being inducible by one or more cytokines, the expression thereof not being limited to hepatocytes.

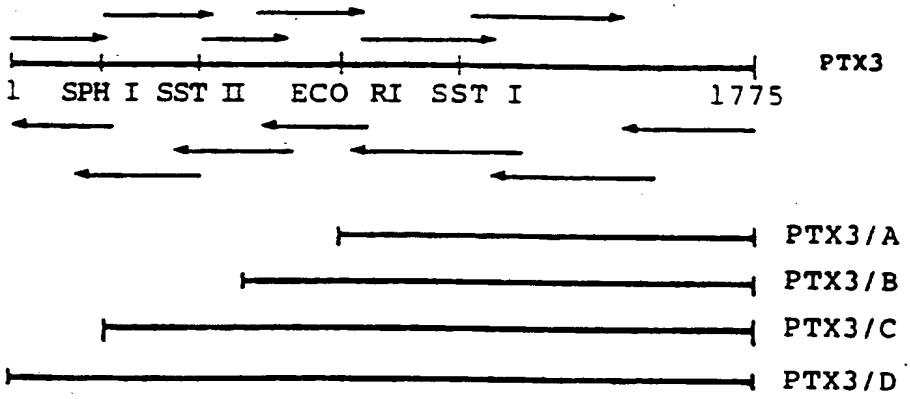

FIGURE 1

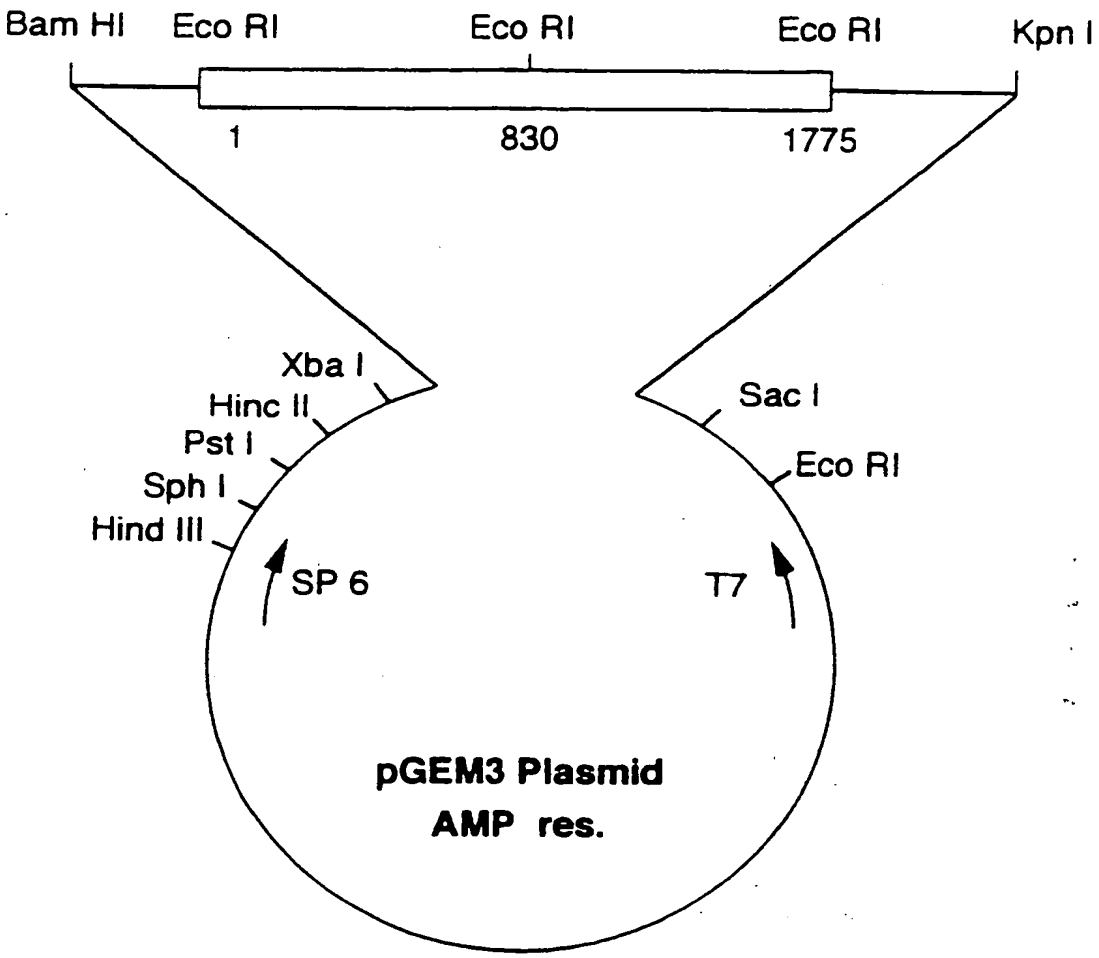

FIGURE 2

FIGURE 3

```
CRP      1 MEK-LLCFLVLTSLSHAFGQTDMSRKAFVFPKESDTSYVSLKAPLTKPLKAFTVCLHFYT  59
SAMP     1 MNKPLLWISVLTSLLEAFAHTDLSGKVFVFPRESVTDHVNLITPLEKPLQNFTLCFRAYS  60
PTX3   179 ----------------------CETAILFPMRSKKIFGSVHPVRPMRLESFSACIWVKA 215
                                  . ....**. * .   .. .   ..*..*. *.   .

CRP        ELSSTRGYSIFSYATKRQDNEILIFWS-KDIGYSFTVGGSEILFEVPEVTVAPVHICTSW 118
SAMP       DLS--RAYSLFSYNTQGRDNELLVYKE-RVGEYSLYIGRHKVTPKVIEKFPAPVHICVSW 117
PTX3       TDVLNKTI-LFSYGTKRNPYEIQLYLSYQSIVFVVGGEENKLVAEAMVSLGRWTHLCGTW 274
           .    ..  .***.*. .  *. .. . .   .    . .. ..       .*.* .*

CRP        ESASGIVEFWVDGK-PRVRKSLKKGYTVGAEASIILGQEQDS--FGGNFEGSQSLVGDIG 175
SAMP       ESSSGIAEFWINGT-PLVKKGLRQGYFVEAQPKIVLGQEQDS--YGGKFDRSQSFVGEIG 174
PTX3       NSEEGLTSLWVNGELAATTVEMATGHIVPEGGILQIGQEKNGCCVGGGFDETLAFSGRLT 334
           .*..*....*..*. . .  ..  *. * .   . .***...   ** *. . .. * ..

CRP        NVNMWDFVLSPDEINTI--YLGGPFSPNVLNWRALKYEVQGEVFTKPQLWP          224
SAMP       DLYMWDSVLPPENILSA--YQGTPLPANILDWQALNYEIRGYVIIKPLVWV          223
PTX3       GFNIWDSVLSNEEIRETGGAESCHIRGNIVGWGVTEIQPHGG----AQYVS          381
           .  .** **. ..* .    . ... *...* . . . .*     .
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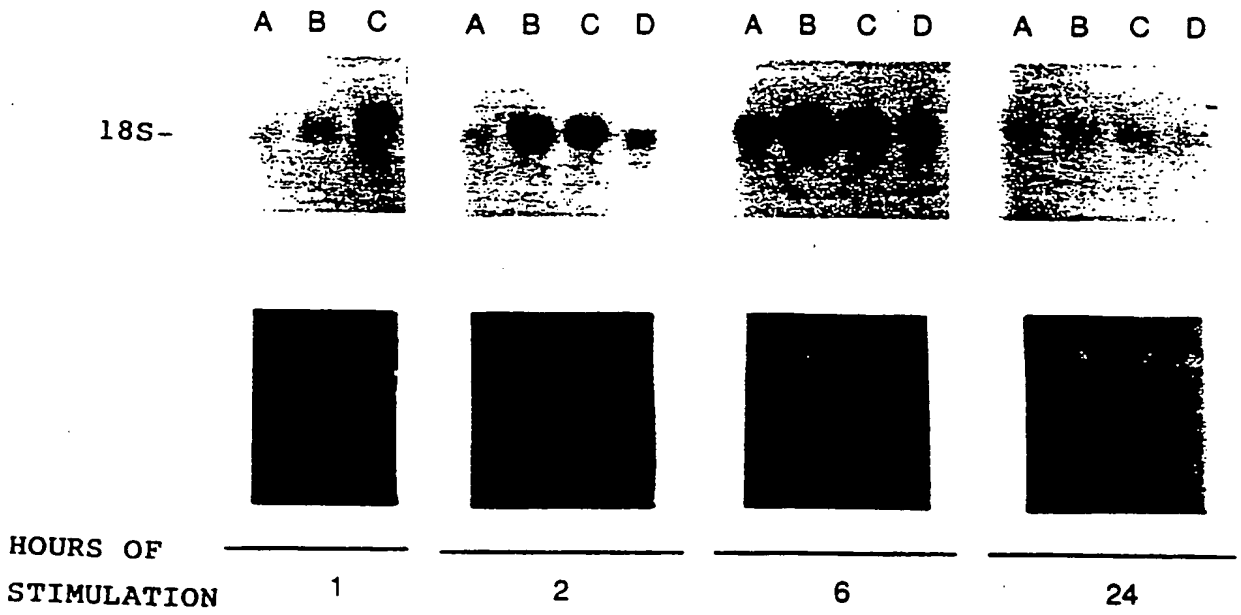

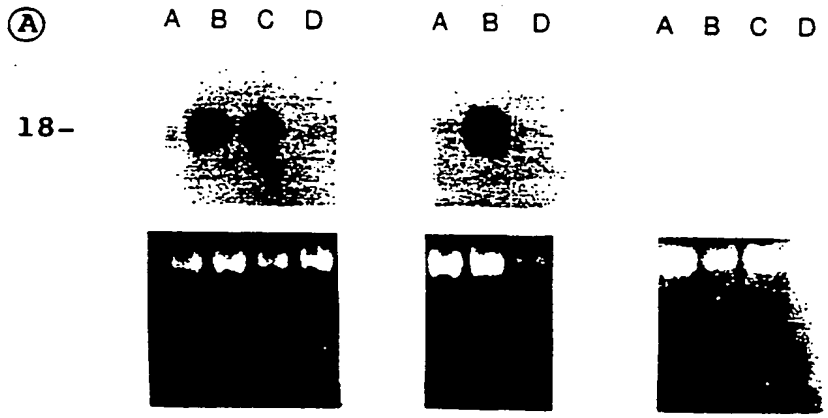

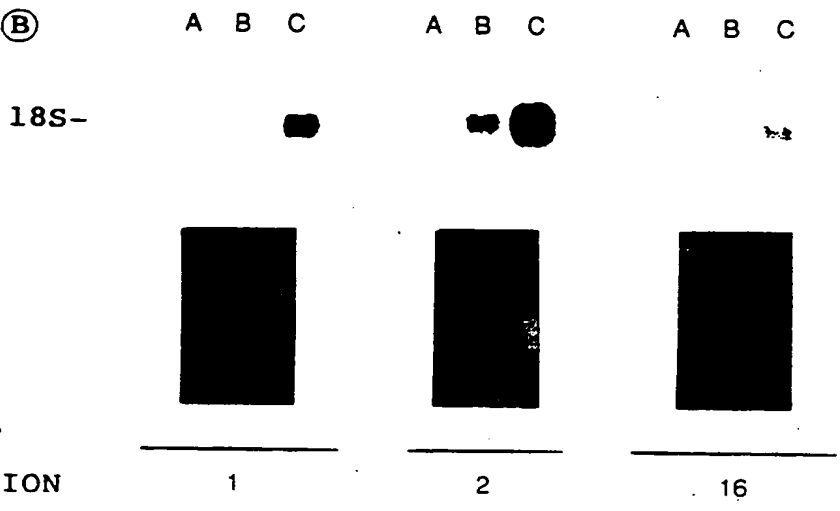

FIGURE 4

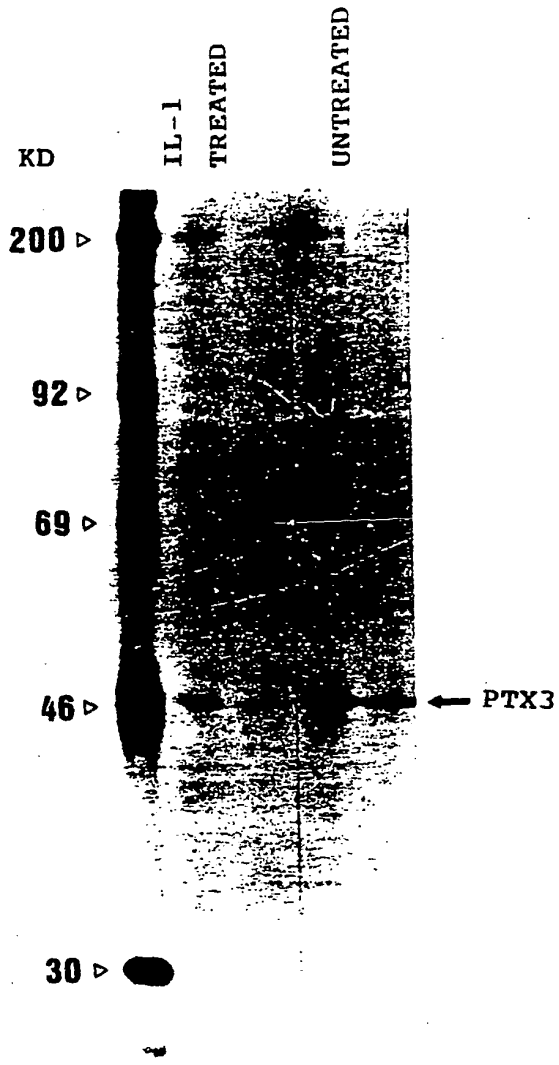

FIGURE 5

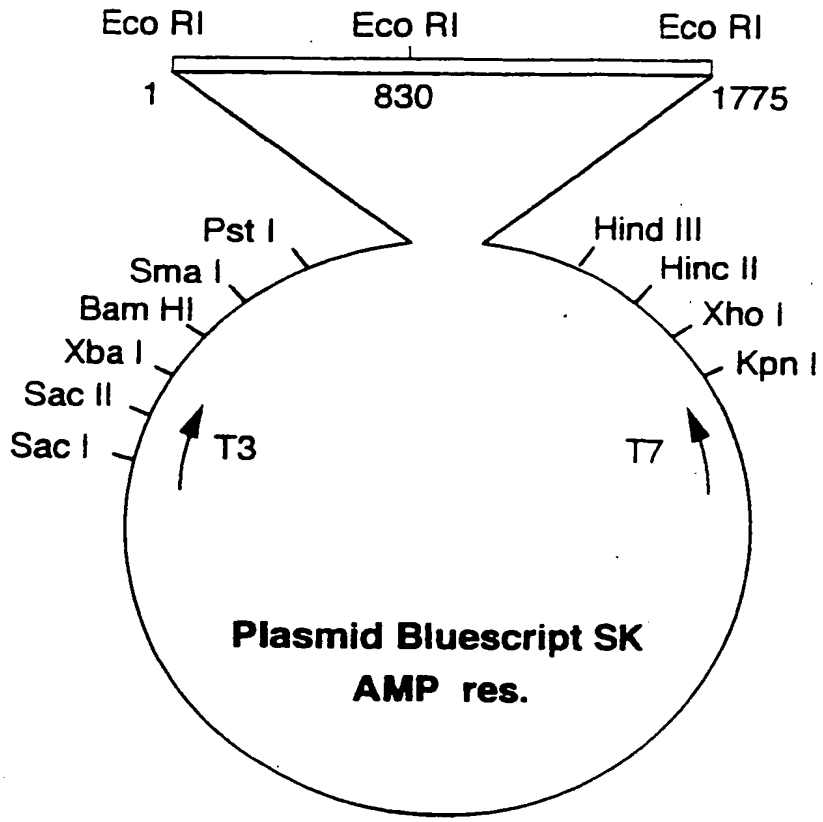

FIGURE 6

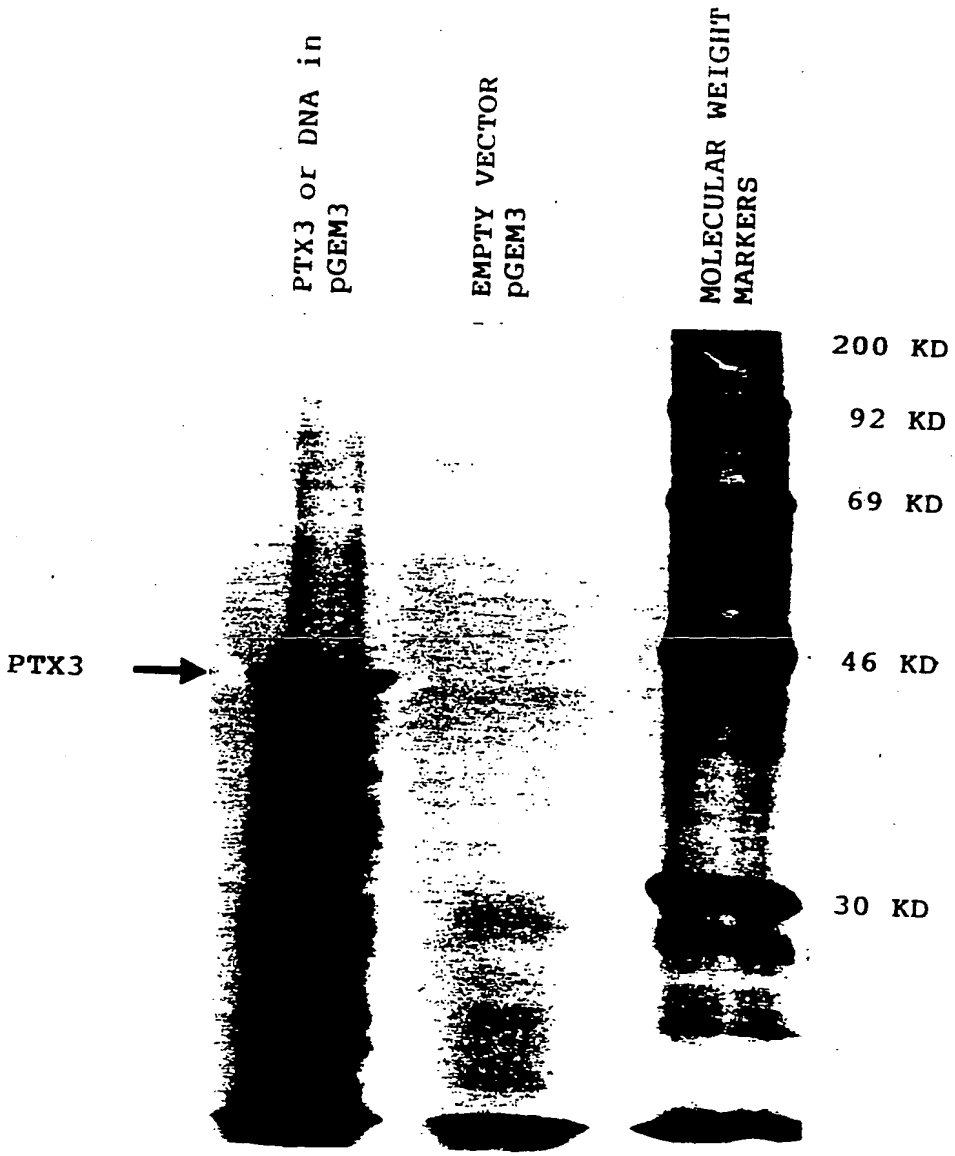

FIGURE 7

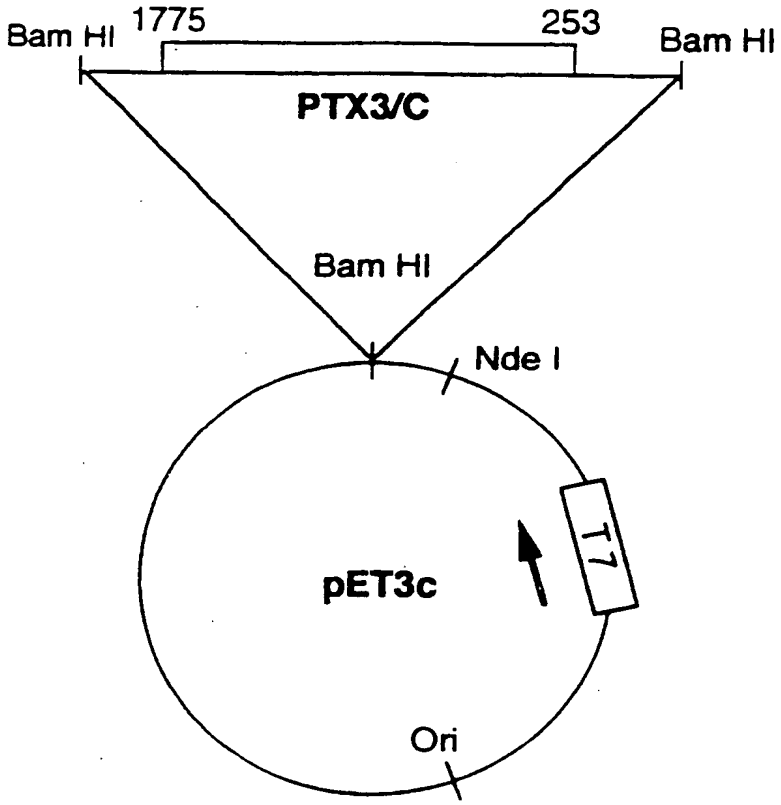

FIGURE 8

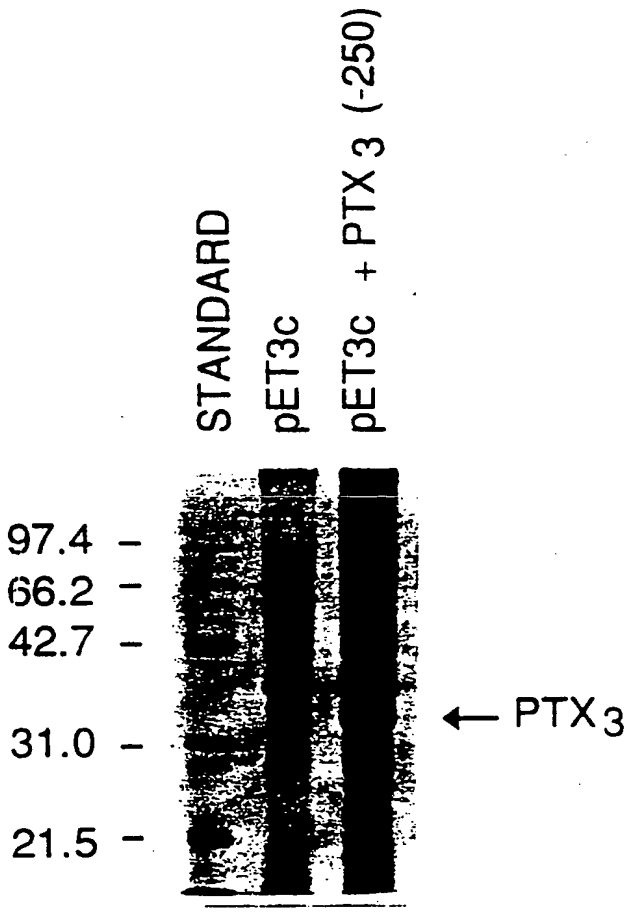

FIGURE 9

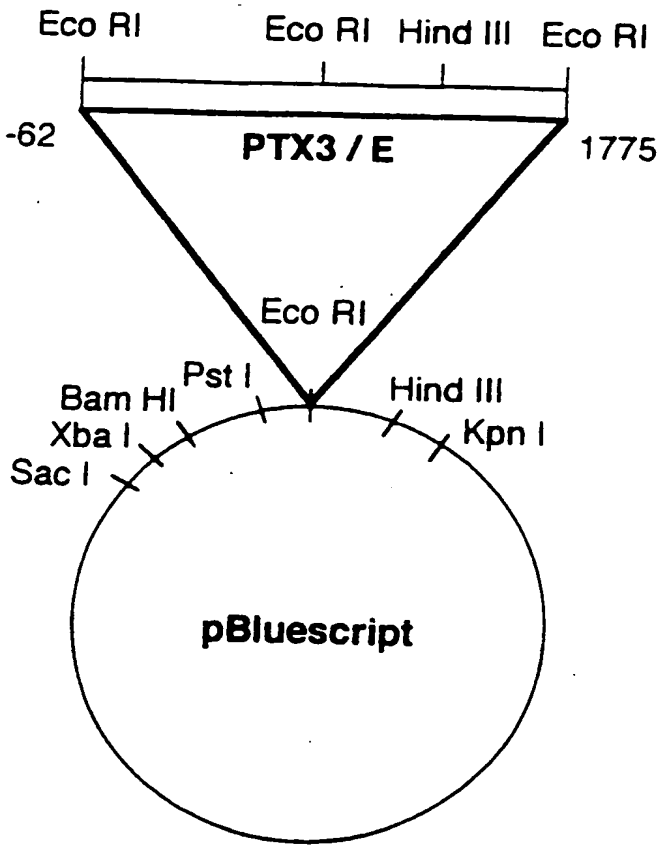

FIGURE 10A

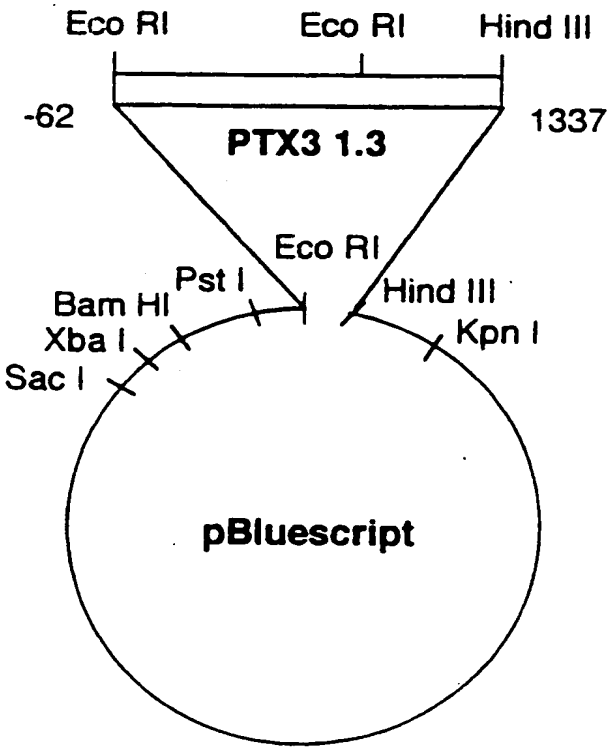

FIGURE 10B

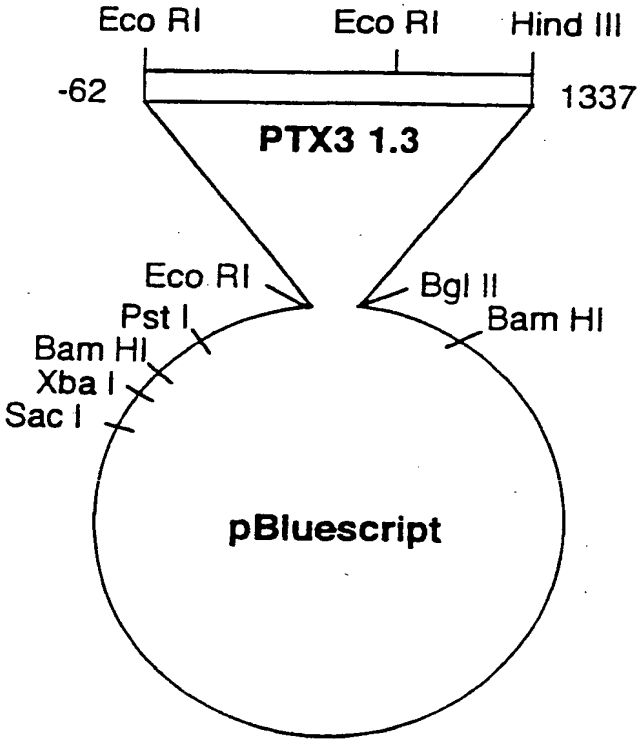

FIGURE 11

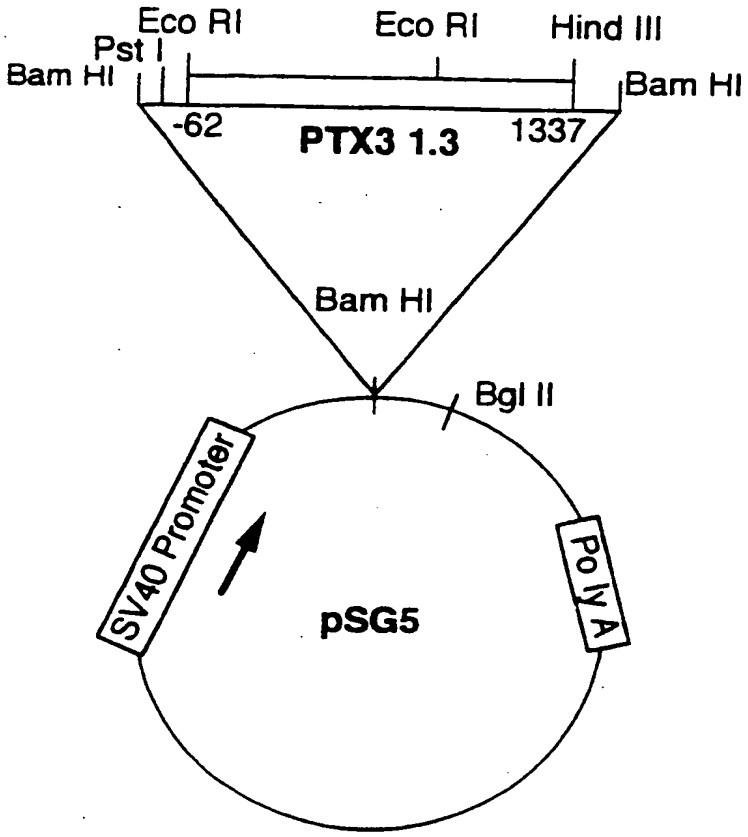

FIGURE 12

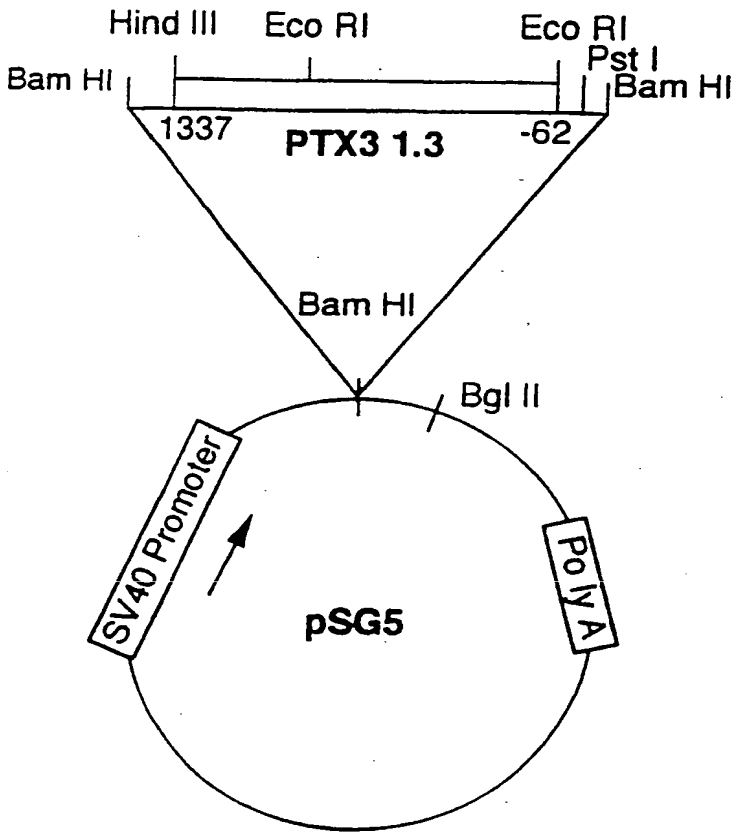

FIGURE 13

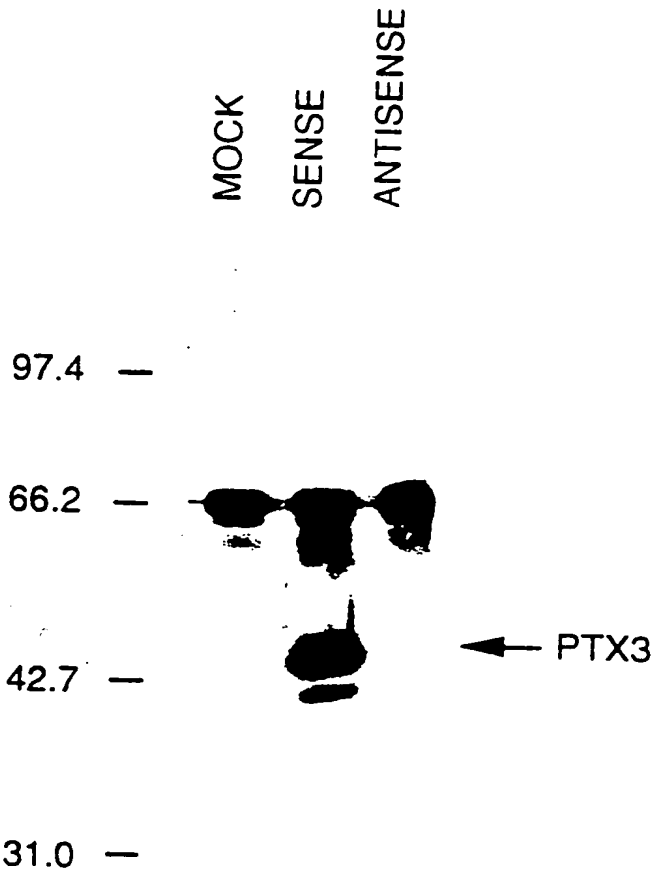

FIGURE 14

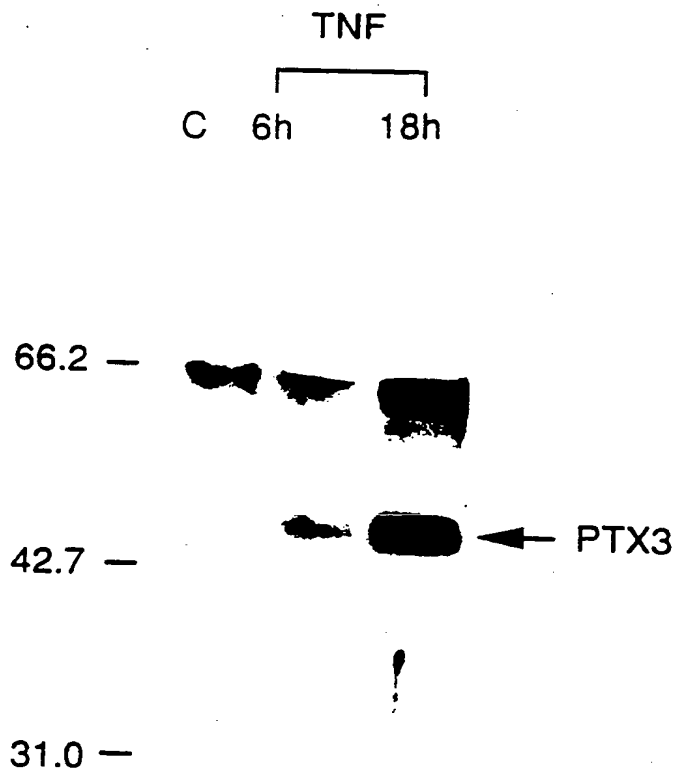

FIGURE 15

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/00868

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C12Q1/68; C12P21/02; //C12N15/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EMBL Database entry HSPTX3R Accession number X63613; 22 January 1992 BREVIARIO, F. ET AL.:'Cloning of a new member of the pentaxin gene family from interleukin-1 stimulated human endothelial cells' see abstract	1-4,13
Y	---	1-9
Y	MOLECULAR AND CELLULAR BIOLOGY vol. 10, no. 5, May 1990, WASHINGTON US pages 1982 - 1988 LEE, T.H. ET AL. 'Isolation and characterization of eight tumor necrosis factor-induced gene sequences from human fibroblasts' cited in the application see the whole document	1-9

	-/--	

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 AUGUST 1993	
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EMBL Database entry HSTSG14A, Accession number M31166 (version 2); 8 December 1991; LEE, T.H. ET AL.: 'Isolation and characterization of eight tumor necrosis factor-induced gene sequences from human fibroblasts' cited in the application see abstract	1-4,13
X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 260, no. 24, 25 October 1985, BALTIMORE, MD US pages 13384 - 13388 WOO, P. ET AL. 'Characterization of genomic and complementary DNA sequence of human C-reactive protein, and comparison with the complementary DNA sequence of serum amyloid P component' cited in the application see the whole document	13
A	THE JOURNAL OF CELL BIOLOGY vol. 116, no. 2, January 1992, pages 545 - 557 LEE, T.H. ET AL. 'A novel secretory tumor necrosis factor-inducible protein (TSG-6) is a member of the family of hyaluronate binding proteins, closely related to the adhesion receptor CD44' see figure 3	
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 31, 5 November 1992, BALTIMORE, MD US pages 22190 - 22197 BREVIARIO, F. ET AL. 'Interleukin-1-inducible genes in endothelial cells' see the whole document	1-13
P,X	WO,A,9 212 176 (NEW YORK UNIVERSITY) 23 July 1992 see the whole document	1-13

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
T	JOURNAL OF IMMUNOLOGY vol. 150, no. 8/II, 15 April 1993, BALTIMORE US page 206A LEE, G.W. ET AL. 'Identification and characterization of TSG-14 , a novel member of the pentaxin family of acute phase proteins' see abstract 1177 & Joint Meeting of the American Association of Immunologists and the Clinical Immunology Society; 21-25 May 1993; Denver, Colorado	1-13

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9300868
SA 72815

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 04/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9212176	23-07-92	AU-A- 1235392	17-08-92

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82